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SOME OBSERVATIONS UPON THE AGGLUTINATION OF BACTERIA.*

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MY purpose in this address is to give briefly some observations upon the value of the agglutination test in establishing the identity or relationship of bacteria and in detecting the variety of bacteria exciting disease in cases of bacterial infection.

During the past three years, in connection with Dr. Katharine R. Collins, I have been more or less occupied in the study of these questions, and in this time I have learned much concerning the difficulties of properly interpreting the results, and of the limitations to the value of the agglutination reaction. It is my hope that a review of some of these experiences may be of interest. Before taking up the discussion of the two topics I wish to touch on some of the points to be thought of in the technique of carrying on the tests.

SOME IMPORTANT POINTS TO CONSIDER IN MAKING AN AGGLUTINATION TEST.

1. The quantitative nature of the union between bacteria and agglutinin. This necessitates that with increase in the number of bacteria in the serum dilution there is more material to combine with the agglutinin. A thick emulsion of bacteria is therefore not agglutinated in as high dilutions as a thin emulsion.

2. The varying sensitiveness of the same variety of bacteria from day to day, even when grown from the same stock culture. At

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times no explanation can be given for this variation, but of its occurrence there can be no doubt.

3. The increased rapidity of union of bacterial substance with agglutinin as the temperature rises from 0° to 37° C. This necessitates that not only the time at which readings are made should be stated, but also whether the reaction took place at the temperature of the ice box, the room, or the incubator.

4. The greater height of the reaction, when long rather than very short periods of time are allowed for its development, provided the test is so carried out that the bacteria do not multiply in the agglutinating fluid.

5. The absence of reaction at times in low dilutions, with presence of reaction in higher dilutions. This phenomenon appears to be due to substances in the serum other than agglutinins. It rarely occurs in dilutions of serum above 1:50.

6. The growth of some varieties of bacteria in the serum-dilutions when the temperature allows of it, thus altering the proportion between bacterial substance and agglutinin. A good reaction may thus disappear in the course of a few hours.

7. The individual judgment in the estimation of what constitutes a certain degree of reaction. No two observers read the completeness of a reaction exactly alike.

8. The alteration in the test serum when it is used over considerable periods of time. A gradual deterioration takes place in the agglutinin in the serum. This is more rapid in diluted serum and with increase of temperature.

9. The medium in which the bacterial suspension is made, whether broth or salt solution, whether it contains sugars or not, etc., is of importance. Growth in glucose media, for instance, makes bacteria more sensitive and tends to natural agglutination. Broth as a medium for diluting serum gives usually a quicker agglutination than does salt solution.

10. The effect of heat and of some preservatives, when they are used, in altering the serum both quantitatively and qualitatively.

11. The considerable difference in the readings made macroscopically and microscopically. A most striking example of the difference in reading is seen in the method used by Dunham[1] and

[1] *Jour. Infect. Dis.*, 1906, Supplement No. 2. p. 10.

that by us. With meningococci examined microscopically after three hours in the incubator we obtained readings of a reaction of 1:200, while Dunham, using light suspensions in the ice chest for 36 hours, obtained a reading of 1:2,000. Both methods were probably equally correct, and the readings by one method could be compared with each other but not with those of the other method.

12. The observation, during the time of experiment, of the control specimen, and of any tendency to natural agglutination.

13. In absorption tests, when filtration methods are employed, the obstruction to the passage of agglutinins of the filter and of the bacteria coating the filter must be fully allowed for, as the coating formed by the various varieties of bacteria differs greatly in permeability.

AGGLUTINATION CHARACTERISTICS AS A GUIDE TO THE CLASSIFICATION AND IDENTIFICATION OF BACTERIA.

It has been unmistakably demonstrated that an agglutinating serum is composed of a number of agglutinins which owe their origin in the animal to the stimulus of the different proteid substances contained in a single cell or in several varieties of cells.

We have many facts which serve to point out the value of partially similar agglutination among bacteria in suggesting relationship such as between certain members of the typhoid-colon group of bacilli. It is true, however, that others which appear just as nearly related do not react to common agglutinins, and some that appear utterly unlike do react.

Thus Durham injected two animals with a different paratyphoid organism. These were obtained from two cases simulating typhoid fever and had the same biochemical activities. He found sera obtained from the two rabbits to have almost no similarity in agglutination. A serum clumping one 1:20,000 did not affect the other in dilutions of 1:100. The marked dissimilarity in the agglutinating characteristics of the bacilli contained in the colon group is another example. Among 14 strains of culturally characteristic colon bacilli isolated by us from 10 persons there were five distinct varieties, if classification were to be made by the agglutinating characteristics. In our recent investigations of pneumococci we have obtained a number of cultures from the exudate of characteristic

cases of lobar pneumonia, which have been alike in morphology, in action on inulin, on sugars, and in cultural characteristics, and yet they have differed absolutely in their affinity for agglutinins. This difference remains unaltered in the cultures as they are continued on culture media, and undoubtedly indicates a different chemical composition; but this is too intangible to be a sufficient reason for separating bacteria which appear to be alike in more essential points. At times, however, it may be very instructive. Thus in Dr. Goodwin's paper on p. 21 it is noted that from the nasal secretion of two healthy students a diplococcus was isolated, which, except in its agglutination, appears to be identical with typical meningococci derived from spinal fluid. This difference in the diplococcus excludes it from the type obtained from the epidemic cases, and even places it under suspicion as to whether it is a meningococcus at all. The complete identity in agglutination characteristics between organisms obtained from the nasal cavity of the sick and from the spinal fluid is strong proof of the former being not only meningococci but the same identical variety as that in the cord.

A species of pathogenic bacteria which develops only in disease is apt to give rise to later generations, all of which will be alike in their agglutinating characteristics, while one which has for the most part a saprophytic life is apt to give rise to distinct varieties. Under semisaprophytic existence the new generations are subjected to variable conditions, and thus become modified, so that, as in the case of the pneumococci, the streptococci, and the colon group of bacilli, we may have a continuance of the more striking cultural characteristics with such variation in the agglutinating affinities as apparently to call for separation into numerous varieties. This separation is useless, so far as we can now see, for any practical purpose, and impossible to define, as there would be no way for future investigators to compare their results unless the original culture or the specific serum was at hand.

The stimulation in an animal of agglutinins for any microorganism is produced, according to our present views, by similar protoplasm in the infecting organisms. Some recent observations,

if correct, indicate that cells apparently widely separated have more or less common substances. Ballner states that a rabbit immunized with a pink yeast developed agglutinins for both typhoid and dysentery bacilli, so that they were agglutinated in dilutions of 1:1,000. A rabbit, immunized by us with yeasts, developed a serum which agglutinated paradysentery bacilli but neither typhoid bacilli or true dysentery bacilli. A less striking experience of our own was that of the serum of a horse, which after immunization with a paradysentery bacillus agglutinated both that bacillus and a typical colon in dilutions of 1:10,000. During injections specific agglutinins are first chiefly developed, but later the total amount of group agglutinins increases so as, at times, to equal the specific ones. The proportional amounts of group agglutinins for allied bacteria differed greatly at different times during the immunization of an animal and at the same time in different animals. Many conflicting statements are due to the lack of appreciation of this variability.

As the quantitative agglutination test usually fails to distinguish whether the reaction is due to specific or group agglutinins, use has been made of an absorption method to determine the action of the specific agglutinin which is present among the multiple agglutinins in the serum of every immunized animal. It has been fairly established that any bacterial strain which can absorb from a serum all the agglutinins which acted upon a certain microorganism, and which were stimulated by that microorganism, must be identical with, or extremely closely allied to, it. The virulence of the two microorganisms may, however, vary widely.

The technique of making the absorption test is rather difficult. When the agglutinating strength of the serum is high, large amounts of bacteria must be added again and again, or the serum must be highly diluted. In the latter case it is impossible to demonstrate that the absorption is complete. Usually the serum is diluted with four times its quantity of salt solution, and then mixed with about its weight of culture. After standing a few hours the mixture is centrifuged. If the supernatant fluid still contains agglutinins, more culture is added and the mixture treated as before. If the agglutinating strength of the serum has not been lowered below that of the

first absorption by the second addition of culture, it is certain that no further absorption by that culture is possible. If a culture is used which is identical with that used in immunization, all agglutinins will be absorbed if sufficient bacteria are added.

When the agglutinating strength is high the organisms may be removed by passage of the fluid through a Berkefeld filter. Here it must be remembered that the filter holds back most of the agglutinin until a number of c.c. have passed, and under certain conditions, as shown by Dunham, the close packing of the bacteria against the filter may continue to hold back agglutinin.

As a general rule it can be said that the agglutinins produced in an animal through the injection of any one variety of bacteria can be exhausted from the serum only by saturating it with sufficient quantities of that variety. In our experience not only the specific, but usually the common, agglutinins stimulatedeby it will thus be absorbed. All other varieties of bacteria will simply absorb any of the common agglutinins for which they have an affinity. If a serum is freed of all common agglutinins, it will clump only the variety of bacteria which was injected in the animal. It is practically impossible to remove absolutely all group agglutinins, since we only know that those having an affinity for the bacteria added have been removed.

The observations of Posselt and v. Sagasser,[1] that by the injections of a pure culture of one variety, agglutinins may be stimulated in large amount for other bacteria, which, however, cannot be absorbed by the variety used in immunization, have not been duplicated by us. The agglutinins not absorbed have been those present in the animal before immunization was begun, or those stimulated by the absorption of other substances. These agglutinins are abundant in horses and goats, especially for the typhoid-colon group. An agglutination of dysentery, paradysentery, and colon bacilli in dilutions of 1:1,000 has been met with by us. Considering our experience, we believe that the absorption test gives most valuable evidence, and much more than does the quantitative test, as to the identity or lack of identity between the bacterium used in immunization and the others tested against its specific serum.

[1] *Wien. klin. Wchnschr.*, 1903, 16, p. 691.

A difficulty frequently met with among recently isolated cultures is their lack of sensitiveness to agglutinins. This is probably due to their growth in blood or in fluid which has been derived from the blood. It is known that the growing of bacteria in a specific serum, and to some extent in any serum, lessens their agglutinability. Thus we cultivated the maltose-fermenting paradysentery bacillus (Flexner, Manila), on each of 11 consecutive days, in fresh broth solutions of the serum from a horse immunized through oft-repeated injections of the bacillus. The solutions used were 1½, 4, and 15 per cent. The serum agglutinated the culture before its treatment in dilutions up to 1:800. After the 11 transfers, the culture grown in the 15 per cent solution ceased to be agglutinated by the serum, and ceased to absorb its specific agglutinins. The cultures grown in the 1½ and 4 per cent solutions agglutinated well in dilutions up to 60 and 100, and continued to absorb agglutinins. The recovery of the capacity to be agglutinated was very slow when the culture was from time to time transplanted on nutrient agar.

It seems that, growing in serum dilutions, the bacteria which developed the least agglutinable substance were least hindered in their growth, and so developed most rapidly. Those producing the least agglutinable substance were thus finally the only ones surviving.

It is sometimes difficult to tell whether a culture is non-agglutinable or simply does not agglutinate in the serum used. An absolute test is to immunize an animal with it and see if it agglutinates in the serum.

THE DEGREE TO WHICH IT IS POSSIBLE TO DETECT THE MICRO-ORGANISM EXCITING A DISEASE BY THE SERUM REACTION OF THE BLOOD OF THE INFECTED PERSON.

The success of the Gruber-Widal test in suspected typhoid fever, cholera, and a few other diseases has given most persons an exaggerated opinion of the diagnostic value of a serum reaction. Even in these diseases the information given by the serum test is not so specific as is thought by many. The serum from typhoid patients occasionally agglutinates one of the varieties of the paratyphoid bacilli in higher dilutions than the typhoid bacilli. In 30 cases tested by us this happened in two instances. Grünberg and Rolly[1]

[1] *Münch. med. Wchnschr.*, 1905, 52, p. 105.

report the remarkable finding, in 40 cases of typhoid fever in which the typhoid bacilli were obtained, that in 35 per cent of the cases, the serum agglutinated a paratyphoid bacillus in higher dilutions than the typhoid bacillus. In these cases it is probably group-agglutinins, excited by the products of certain colon bacilli secondarily infecting the Peyer's patches, which agglutinated the paratyphoids, rather than the group agglutinins due to the typhoid bacilli.

As the clinician, when considering a case of continued fever, is as a rule trying to settle whether it is one of tuberculosis, malaria, or typhoid fever, he is satisfied to know whether the infection is or is not due to one of the typhoid-colon group, and does not mind the impossibility of an absolute identification of the variety. In the case of dysentery, a quantitative agglutination test is frequently useless as an indication whether the dysentery or paradysentery bacilli are exciting the disease. In cases due to the Shiga bacillus, the serum occasionally agglutinates one of the mannite-fermenting dysentery types in higher dilutions than itself. This again is probably due to group agglutinins produced by the absorption of substances contained in certain varieties of colon bacilli. A goat injected by us with a colon bacillus produced a serum which agglutinated it in a 1:5,000 dilution, and agglutinated the paradysentery bacilli in a dilution as high as 1:2,500. Agglutination of the Shiga bacilli by a serum which does not agglutinate the paradysentery bacilli usually indicates infection with the former variety, but an agglutination of the paradysentery bacilli alone may indicate a colon infection. Different members of groups of bacteria, like the colon group or the pneumococci, though having common pathogenic properties, frequently differ almost absolutely in their reaction to agglutinins. Thus, a sheep injected by us with a typical pneumococcus agglutinated that organism in a 1:100 dilution but did not agglutinate 20 other pneumococci in dilutions higher than 1:2. An equal variation was found by us to exist among the members of the colon group of bacilli. In infections which may be due to any one of a number of varieties differing in their agglutination characteristics, it is almost impossible to use sufficient cultures to diagnose by the serum reaction whether one of the group was the exciting factor.

The greatest limitation to the use of the serum reaction is the fact that the majority of bacteria do not, in the course of an infection, excite a sufficient amount of agglutinin to be readily detected, as for instance in the case of tubercle, influenza, and diphtheria bacilli.

Bacteria widely separated may, in exciting great quantities of agglutinin for themselves, develop so much group agglutinin for each other as to be misleading. An animal injected with staphylococcus agglutinated the typhoid bacillus in 1:160, while before, only 1:10. Another, injected with B. proteus, agglutinated a culture of this in 1:160,000, and also the typhoid bacillus in 1:1,200. In such a case, if the typhoid bacillus was suspected as the cause of the infection as above tested, the serum reaction would be apt to deceive.

In actual natural infections such very high reactions are improbable, but those sufficiently high to give misleading group reactions frequently occur. In adults the blood is apt to contain a considerable amount of group agglutinins for many bacteria before the special infection which is to be investigated developed. It is only through long experience that we are able to determine in how high dilutions such agglutinins are apt to act, and therefore in what dilutions a specific reaction can be suspected or considered proven. In suspected typhoid infection, for instance, we are now able to state that a reaction in a 1:50 dilution in two hours at room temperature is proof of an infection with a member of the typhoid-colon group, and as the great majority of such infections are due to the typhoid bacillus, we can consider this as the probable microorganism. Agglutination of the typhoid bacillus in higher dilutions makes this probability almost, but not quite, a certainty.